US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for Neutralization Confirmation Assay For Disinfectant (Liquid or Spray) Products Tested Against *Mycobacterium bovis* (BCG)

SOP Number: MB-11-02

Date Revised: 09-04-07

Superseded SOP: MB-11-01 Neutralization Confirmation Assay For Disinfectant (Liquid or Spray) Products Tested Against *Mycobacterium bovis* (BCG)

EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

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for
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Date Revised: 09-04-07

Initiated By: _____ Date: ___/___/___

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Effective Date: ___/___/___

Controlled Copy No.: _____

Withdrawn By: _____ Date: ___/___/___

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1.0 SCOPE AND APPLICATION:

1.1 The neutralization of the active ingredients found in antimicrobial products is one of the most important steps in efficacy testing. A neutralizing agent is used in each test to inactivate the product's active ingredients, a process essential to achieving the desired contact time. In addition, the neutralizer itself or in combination with the recovery medium must not exhibit bacteriostatic activity against the test microbe; bacteriostatic activity may bias the efficacy data.

1.2 This SOP describes methodology which will be used to determine the effectiveness of neutralizers specified for efficacy tests of hard surface disinfectants against *Mycobacterium bovis* (BCG).

1.3 This method can also be used to determine the effectiveness of an alternative neutralizer, one not specified in the test parameters.

1.4 It is preferable to perform the neutralization assay concurrently with product testing; however, an independent, stand-alone assay may also be performed. The neutralization assay should be performed on at least one of the two lots of product tested for efficacy.

2.0 DEFINITIONS:

2.1 AOAC = AOAC INTERNATIONAL

2.2 CTB = Confirmatory Tuberculocidal Test

2.3 MPB = Modified Proskauer Beck

2.4 M7H9 = Middlebrook 7H9 Agar and Broth

2.5 K = Kirchners Medium

2.6 TB = TB Broth

2.7 CFU = Colony Forming Unit

2.8 PBDW = Phosphate Buffered Dilution Water

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organism are required to be performed in accordance

with biosafety practices stipulated in the SOP MB-01 (see ref. 15.1). All manipulations of *M. bovis* (BCG) are performed in a biosafety level 3 laboratory (e.g. room B202 or room B207).

3.2 Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Latex gloves and other personal protective clothing or devices must be worn during the handling of these items for purposes of activation or dilution, or efficacy testing. A chemical fume hood or other containment equipment is employed when performing tasks with concentrated products.

4.0 CAUTIONS:

4.1 To ensure the stability of the test disinfectant, prepare the disinfectant dilutions within three hours of the disinfectant treatment step unless test parameters specify otherwise.

4.2 Strict adherence to the protocol is necessary for validity of test results.

4.3 Use aseptic procedures for all test procedures involving manipulations of the test organisms and associated test components.

5.0 INTERFERENCES:

5.1 For each assay, one batch (preparation) of each medium should be used for both treatment and control groups. Differences in performance (quality) between batches of media may lead to misleading neutralization results.

5.2 Presence of contamination (i.e. in media controls) will interfere with the interpretation of results and may necessitate repeat analysis.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 Refer to section 7, SPECIAL APPARATUS AND MATERIALS, of each efficacy test method SOP cited in this document.

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Refer to section 8, INSTRUMENT OR METHOD CALIBRATION, of each efficacy test method SOP cited in this document.

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Disinfectants are stored according to manufacturers' recommendations or at room temperature if the product label or testing parameters do not identify a storage temperature. Those disinfectants requiring activation or dilution prior to use will only be activated or diluted within three hours of testing unless test parameters specify otherwise.

10.0 PROCEDURE AND ANALYSIS:

10.1 General Description of the Assay. The general procedure for conducting the assay is the same for liquid and spray products. Most importantly, the test parameters specified for product testing (e.g., H_2O hardness, Use Dilution, Organic Soil, Neutralizer, Contact Time, Temperature) must also be followed for the neutralization confirmation assay.

In brief, this assay is designed to simulate the conditions of the efficacy test; however, sterile carriers are used instead of inoculated carriers. Diluted *M. bovis* BCG inoculum is added directly to the various sets of subculture media tubes (see Table 1, p. 10). The inoculum is quantified by plating on M7H9 agar. This provides for a quantitative approach to assessing the effectiveness of the neutralizer and any possible bacteriostatic action resulting from the neutralizer/subculture media/disinfectant carryover combinations.

10.2 Preparation of Inoculum. In this assay, the inoculum is standardized by diluting the pure culture to yield 20.0% ±1.0% transmittance at 650 nm; dilutions of the standardized inoculum are applied directly to tubes of subculture media as described in section 10.4.10. The term “inoculated” is used to describe this process.

10.2.1 *M. bovis* BCG cultures used in neutralization assays are generated in the same fashion as the cultures used for other tuberculocidal assays. Culturing steps are described in section 10.1 of SOP MB-07-03 (see ref. 15.4).

10.2.2 For neutralizer assays conducted concurrently with product testing,

use the standardized inoculum prepared for inoculating carriers.

10.2.3 If the neutralizer assay is conducted independently of a product test, harvest, homogenize and allow culture to settle 10-15 minutes; then, remove the culture remaining in suspension and standardize (20.0% ±1.0% transmittance at 650 nm) the *M. bovis* BCG culture as described in sections 10.1.6 to 10.1.10 of SOP MB-07-03 (see ref. 15.4). Record this information on the Neutralization Confirmation Assay: Time Recording Sheet for *M. bovis* (BCG) Inoculum Preparation (see 16.0).

10.2.4 If the product test parameters specify the addition of an organic soil load to the inoculum, then the neutralization assay will be performed with the organic soil load added to the inoculum. Otherwise, the inoculum should be prepared without the addition of an organic soil load.

10.2.5 Initiate serial ten-fold dilutions of the inoculum by pipetting 1 mL of the standardized broth culture into 9 mL of PBDW. Four dilutions (10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}) will be used to inoculate the subculture media described below. The target number of cells to be delivered per tube is 5-100 CFU/mL; this level should be seen in one of the two highest dilutions.

10.2.6 To estimate CFU/mL, plate (spread plate or pour plate method) each of the four dilutions in duplicate on M7H9 agar. Briefly vortex each dilution tube prior to plating. Plate 0.1 mL aliquots of dilutions 10^{-4} thru 10^{-7} (for spread plating). One mL aliquots of 10^{-5} thru 10^{-8} are added for pour plating methods.

10.2.7 Record the dilution and plating information on the Neutralization Confirmation Assay: Serial Dilution/Plating Tracking Form for *M. bovis* (BCG) (see 16.0).

10.2.8 If the spread plate method is used for bacterial enumeration, M7H9 agar plates are prepared in advance and are refrigerated prior to use to avoid possible contamination. Bring refrigerated plates to room temperature before dispensing the appropriate volume of dilutions to the surface of the agar. The inoculum from the respective dilutions is spread over the medium surface.

10.2.9 If the pour plate method is used for bacterial enumeration, the M7H9 agar is prepared and tempered (approx. 1 hr) to 45-50°C in a waterbath prior to use. Tempered M7H9 agar is added to the plate after the addition of the appropriate dilution, and swirled to spread the inoculum.

10.2.10 Incubate plates at 36±1°C for 21-25 days. Count colonies with aid of a plate counter. Plates that have colony counts over 300 can be estimated or labeled TNTC. Record the counts on the Neutralization Confirmation Assay: Inoculum Enumeration Form for *M. bovis* (BCG) (see 16.0).

10.3 Disinfectant Sample Preparation.

10.3.1 Follow guidelines for disinfectant sample preparation provided in the appropriate test method SOP.

10.4 Performing the Assay. The following instructions apply to the analysis of one neutralizer with one product sample.

10.4.1 Each assay will require eight sterile carriers, four for the Primary Subculture Treatment and four for the Subculture Media + Fresh neutralizer Control. One carrier is used per each inoculum dilution described in 10.2.5. Use the carrier type required for the product (i.e. porcelain penicylinders for liquid products or glass slide carriers for spray products).

10.4.2 Four sterile carriers will be exposed to disinfectant; each will be exposed to the disinfectant according to the contact time specified in the test parameters for efficacy testing. The product must be applied according to specific instructions (e.g., use-dilution, contact time, spray distance, spray period) provided in the test parameters. Procedures for treating carriers with liquid and spray disinfectants are described in the OPP Microbiology Laboratory SOPs: MB-07 (see ref. 15.4), and MB-06 (see ref. 15.3), respectively.

10.4.3 As with product testing, expose the carriers to disinfectant at 30 seconds or one minute intervals (± 5 seconds of the exact time of the treatment). If a specific contact time is stipulated by the manufacturer other than 10 minutes, the interval is modified to accommodate their claims. Record the carrier transfer information

on the Neutralization Confirmation Assay: Time Recording Sheet for *M. bovis* (BCG) Inoculum Preparation (see 16.0)

10.4.4 Allow product to remain on the carrier per the specified contact time.

10.4.5 After the last carrier of the set (first 4 carriers) has been treated with the disinfectant, and the contact time is complete, aseptically transfer carriers in order in a timed fashion into tubes containing the specified neutralizer within the ± 5 seconds time limit. Transfer carriers according to methods specified in the appropriate efficacy test method SOP. Drain the excess disinfectant from the carrier prior to the transfer.

Note: For spray products, the amount of neutralizer is 20 mL per tube (38 x100 mm Bellco tubes) compared to 10 mL (25 x100 mm tubes) used in the CTB test method for liquid products.

10.4.6 Thoroughly shake the neutralizer tube with the carrier in it and immediately transfer each carrier to a culture tube containing the primary subculture medium (i.e. MPB). This set of MPB tubes (4 total tubes) will represent the **Primary Subculture** Treatment. Each tube will be inoculated with one of the four inoculum dilutions (see sections 10.4.10 and 10.4.11).

10.4.7 From each neutralizer tube (4 total), transfer 2 mL aliquot of neutralizer to one tube of each subculture medium (M7H9, K or TB) specified by the test parameters. This portion of the assay is not timed, but the aliquots should be delivered to the subculture media as quickly as possible. This set of eight tubes (4 tubes of each of the two subculture media) represents the **Secondary Subculture + Exposed Neutralizer** (exposed to the disinfectant) Treatment. One tube of each medium will be inoculated with one of the four inoculum dilutions (10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}), see sections 10.4.10 and 10.4.11.

Note: For spray products, eight tubes of each of the additional subculture media (not MPB) specified will each be inoculated with 2 mL of the exposed neutralizer. Duplicate tubes for each additional subculture media will be inoculated with one of the four inoculum dilutions (10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}).

10.4.8 **Subculture Media + Fresh Neutralizer** Control. The four remaining sterile carriers will be exposed to neutralizer ONLY one carrier per tube of neutralizer) and transferred aseptically to four tubes of MPB. Thoroughly shake the neutralizer tube with the carrier in it and immediately transfer each carrier to a culture tube containing MPB. From each neutralizer tube (4 total), transfer 2 mL of neutralizer to one tube of each subculture medium (M7H9, K or TB). This portion of the assay is not timed, but the aliquots should be delivered to the subculture media as quickly as possible. One tube of each medium will be inoculated with one of the four inoculum dilutions (10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}), see section 10.4.10.

Note: For spray products, eight tubes of each of the additional subculture media specified (not MPB) will each be inoculated with 2 mL of the exposed neutralizer. Duplicate tubes for each additional subculture media will be inoculated with one of the four inoculum dilutions (10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}).

10.4.9 **Subculture Media Only** Control. This control contains four tubes of each preparation of subculture media used in the neutralization assay. Neutralizer is not added to the media. Each tube of each medium will be inoculated with one of the four inoculum dilutions (see section 10.4.10).

10.4.10 Inoculating Subculture Media. Inoculate each treatment and control tube with 1 mL of the diluted *M. bovis* (BCG) inoculum as indicated in Table 1. Inoculate the tubes following the transfer of all carriers and neutralizer. In addition, refer to Table 2 (p. 15) and Table 3 (p. 16) for organization of neutralization assays for liquid, and spray products, respectively.

Table 1. Inoculating Treatments and Control Groups with diluted *M. bovis* (BCG) culture[a]

Primary Subculture Treatment	Secondary Subculture Media + Exposed Neutralizer Treatment	Subculture Media + Fresh Neutralizer Control	Subculture Media Only Control
MPB with carrier: 1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4	M7H9: 1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4	MPB with carrier: 1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4	MPB: 1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4
	K or TB: 1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4	M7H9: 1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4	M7H9: 1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4
		K or TB: 1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4	K or TB: 1 mL of 10^{-5} → Tube 1 1 mL of 10^{-6} → Tube 2 1 mL of 10^{-7} → Tube 3 1 mL of 10^{-8} → Tube 4

[a] For spray products, 20 mL of MPB are placed in 38 x 100 mm tubes. For Secondary Subculture Media + Exposed Neutralizer Treatment and Subculture Media + Fresh Neutralizer Control, use 2 tubes of each medium (M7H9, K or TB) per dilution. Both media (M7H9, K or TB) receive 2 mL aliquots of neutralizer.

10.4.11 **Negative (uninoculated)** media controls (one tube of each medium) will also be included.

10.4.12 Incubate all tubes for 60 days at 36±1°C. If no growth or occasional (insufficient for confirmation) growth occurs, incubate an additional 30 days before recording final results.

10.5 Results are recorded as positive (+) or negative (0) as indicated by the presence or absence of growth. Prior to entering (+) or (0), an acid fast stain is performed. Record results at 60 days and again at 90 days if necessary.

10.6 Identification and Confirmation Testing:

10.6.1 The confirmatory tests used to verify the identity of *M. bovis* (BCG) are acid fast staining and plating on selective media.

10.6.2 For each medium in each of the treatment and control groups, select the tube with growth from the highest dilution of inoculum (fewest CFU/mL delivered) and perform acid fast staining on a sample of the growth. Acid fast rods are typical for *M. bovis* (BCG).

10.6.3 Additional confirmation can be conducted if necessary to include plating onto selective media such as Middleblook 7H9 agar plates.

10.6.4 Following the 21-25 day incubation period, the colony morphology of the organism on M7H9 agar should be evaluated. *M. bovis* (BCG) typically appears as colorless to buff-colored, raised, rough growth on M7H9 agar.

10.6.5 Record confirmation results on the Neutralization Confirmation Assay: Test Microbe Confirmation Sheet for *M. bovis* (BCG) (see 16.0).

10.7 Interpretation of Results.

10.7.1 Review the plate count data. The plate counts are an essential element of this assay. One of the four dilutions plated should provide counts within the target range, 5-100 CFU/mL. Subculture tubes inoculated from this dilution also received this low level of challenge, an aspect critical to the determination of neutralization effectiveness and bacteriostatic activity.

10.7.1.1 The lack of complete neutralization of the disinfectant or bacteriostatic activity of the neutralizer itself may be masked when a high level of *M. bovis* (BCG) is added to the subculture tubes.

10.7.2 The **Subculture Media Only** Control tubes provide a basis for comparison of growth in the subculture tubes of other treatments. Growth in Media Control tubes verifies the performance of the media and the presence of *M. bovis* (BCG) in the diluted inoculum.

No growth or only growth in tubes which received high levels of inoculum (e.g., a dilution with plate counts which are too numerous to count) indicates poor media performance.

10.7.3 The occurrence of growth in the **Subculture Media + Fresh Neutralizer Control** tubes is used to determine whether there are any bacteriostatic effects attributed to possible interactions between the neutralizer + subculture media. Interactions between the media and neutralizer may result in growth in some media and not others.

No growth or growth only in tubes which received a high level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates the presence of bacteriostatic properties as a result of the neutralizer-media interaction.

10.7.4 The occurrence of growth in the **Secondary Subculture/Exposed Neutralizer** treatment tubes is used to determine the effectiveness of the neutralizer against the disinfectant when used under simulated test conditions. Interactions between the media and neutralizer may result in growth in some media and not others.

No growth or growth only in tubes which received a high level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates ineffective neutralization and/or the presence of bacteriostatic properties as a result of the neutralizer-media interaction.

10.7.5 The occurrence of growth in the **Primary Subculture Treatment** tubes is also used to determine the effectiveness of the neutralizer against the disinfectant when used under simulated test conditions.

No growth or growth only in tubes which received a high level of inoculum (e.g., the dilution with plate counts which are too numerous to count) indicates ineffective neutralization and/or the presence of bacteriostatic properties as a result of the neutralizer-media interaction.

10.7.6 Each tube of **negative (uninoculated)** media controls must show no growth.

11.0 DATA ANALYSIS/CALCULATIONS: None

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

Data will be recorded promptly, legibly, and in indelible ink on testing forms (see. 16.0). Completed forms are archived in notebooks kept in secured file cabinets in D217. Only authorized personnel have access to the secured files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03-02 (see ref. 15.2).

13.0 QUALITY CONTROL:

13.1 The OPP Microbiology Laboratory conforms to 40 CFR Part 160, Good Laboratory Practice Standards. Appropriate quality control measures are integrated into each SOP.

13.2 For quality control purposes, the required information is documented on the appropriate form(s) (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Any deviation from the standard protocol and the reason for the deviation will be recorded on the appropriate record sheet (see 16.0); corrective action will be expeditious.

15.0 REFERENCES:

15.1 MB-01: Biosafety in the Laboratory

15.2 ADM-03: Records and Archives

15.3 MB-06: Testing of Spray Disinfectants Against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Mycobacterium bovis* (BCG)

15.4 MB-07: Confirmatory Tuberculocidal Method for Testing Disinfectant Efficacy

16.0 FORMS AND DATA SHEETS:

16.1 Neutralization Confirmation Assay: Time Recording Sheet for *M. bovis* (BCG) Inoculum Preparation

16.2 Neutralization Confirmation Assay: Time Recording Sheet for Carrier Transfers for *M. bovis* (BCG)

16.3 Neutralization Confirmation Assay: Information Sheet for *M. bovis* (BCG)

16.4 Neutralization Confirmation Assay: Results Sheet for *M. bovis* (BCG)-Liquid Products

16.5 Neutralization Confirmation Assay: Results Sheet for *M. bovis* (BCG)-Spray Products

16.6 Neutralization Confirmation Assay: Serial Dilution/Plating Tracking Form for *M. bovis* (BCG)

16.7 Neutralization Confirmation Assay: Inoculum Enumeration Form for *M. bovis* (BCG)

16.8 Neutralization Confirmation Assay: Test Microbe Confirmation Sheet for *M. bovis* (BCG)

Table 2: Components of the Neutralization Confirmation Assay For Liquid Products

Treatment/Control	*M. bovis* BCG Inoculum Dilution (1 mL added per tube)	Media (○ = Tube of Media)		
		MPB (20 mL)	M7H9 (20 mL)	K or TB (20 mL)
Primary Subculture	10^{-5}	○/Carrier	No Tube	No Tube
Primary Subculture	10^{-6}	○/Carrier	No Tube	No Tube
Primary Subculture	10^{-7}	○/Carrier	No Tube	No Tube
Primary Subculture	10^{-8}	○/Carrier	No Tube	No Tube
Secondary Subculture + Exposed Neutralizer	10^{-5}	No Tube	○	○
Secondary Subculture + Exposed Neutralizer	10^{-6}	No Tube	○	○
Secondary Subculture + Exposed Neutralizer	10^{-7}	No Tube	○	○
Secondary Subculture + Exposed Neutralizer	10^{-8}	No Tube	○	○
Subculture Media + Fresh Neutralizer Control	10^{-5}	○/Carrier	○	○
Subculture Media + Fresh Neutralizer Control	10^{-6}	○/Carrier	○	○
Subculture Media + Fresh Neutralizer Control	10^{-7}	○/Carrier	○	○
Subculture Media + Fresh Neutralizer Control	10^{-8}	○/Carrier	○	○
Media Only Control	10^{-5}	○	○	○
Media Only Control	10^{-6}	○	○	○
Media Only Control	10^{-7}	○	○	○
Media Only Control	10^{-8}	○	○	○
Uninoculated Control	Not inoculated	○	○	○

Table 3: Components of the Neutralization Confirmation Assay For Spray Products

Treatment/Control	*M. bovis* BCG Inoculum Dilution (1 mL added per tube)	Media (○ = Tube of Media)		
		MPB (20 mL)	M7H9 (20 mL)	K or TB (20 mL)
Primary Subculture	10^{-5}	○/Slide Carrier	No Tube	No Tube
Primary Subculture	10^{-6}	○/Slide Carrier	No Tube	No Tube
Primary Subculture	10^{-7}	○/Slide Carrier	No Tube	No Tube
Primary Subculture	10^{-8}	○/Slide Carrier	No Tube	No Tube
Secondary Subculture + Exposed Neutralizer	10^{-5}	No Tube	○○	○○
Secondary Subculture + Exposed Neutralizer	10^{-6}	No Tube	○○	○○
Secondary Subculture + Exposed Neutralizer	10^{-7}	No Tube	○○	○○
Secondary Subculture + Exposed Neutralizer	10^{-8}	No Tube	○○	○○
Subculture Media + Fresh Neutralizer Control	10^{-5}	○/Slide Carrier	○○	○○
Subculture Media + Fresh Neutralizer Control	10^{-6}	○/Slide Carrier	○○	○○
Subculture Media + Fresh Neutralizer Control	10^{-7}	○/Slide Carrier	○○	○○
Subculture Media + Fresh Neutralizer Control	10^{-8}	○/Slide Carrier	○○	○○
Media Only Control	10^{-5}	○	○	○
Media Only Control	10^{-6}	○	○	○
Media Only Control	10^{-7}	○	○	○
Media Only Control	10^{-8}	○	○	○
Uninoculated Control	Not inoculated	○	○	○

Neutralization Confirmation Assay: Time Recording Sheet for *M. bovis* (BCG) Inoculum Preparation

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Type of Product (circle one)	Liquid Spray
Product Reg. No.	
Product Name	
Sample No(s).	
Neutralizer	

Date/Initials		
Inoculum Settle Time (from clock/and a timer)		Test Culture %T
Start Time	End Time	
/	/	
/	/	
Comments:		

Neutralization Confirmation Assay: Time Recording Sheet for Carrier Transfers for *M. bovis* (BCG)

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Product Reg. No.	
Product Name	
Sample No(s).	
Neutralizer	

Initials/ date	Drop, or Spray Interval	Carrier Drop or Spray Start Time (application of the disinfectant)		Carrier Drop or Spray End Time (transfer to neutralizer then to MPB Tube)		Transfer of Neutralizer to Additional Subculture Media
		Clock	Timer (target)	Clock	Timer (target)	Start Time[1]
Analyst Dropping or Spraying Carriers:						
Analyst Transferring Neutralizer into Subculture Media:						
Comments:						

1 Transfer of neutralizer into secondary subculture taken from the clock.

Neutralization Confirmation Assay: Information Sheet for *M. bovis* (BCG)

OPP Microbiology Laboratory

PRODUCT INFORMATION/Confirmed by:_____			
EPA Reg. No.		Sample No.	
Name		Test Date	

PARAMETERS FOR PRODUCT TESTING/Confirmed by:_____			
H_2O Hardness ($CaCO_3$) ppm	Specified	Titrated (Buret)/Date/Init	HACH/Date/Init
		/ /	/ /
Use Dilution	Specified	As Prepared/Date/Init	
		/ /	
Organic Soil	Specified	As Prepared/Date/Init	
		/ /	
Neutralizer	Specified		
Temperature	Specified	Chiller Display	Test tube Waterbath
		Before: After:	Before: After:
Contact Time	Specified	As Tested	
Other Parameters	Specified		

TEST MICROBE INFORMATION/Confirmed by:_____			
Org. Control No.		21-25 Day *M. bovis* (BCG) Culture	
% Transmittance		Date/Time Initiated:	Date/Time Harvested:

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Neutralization Confirmation Assay: Results Sheet for *M. bovis* (BCG)-Liquid Products

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Product Name		Neutralizer	
Sample No.		Comments	

TEST RESULTS: Date Recorded/Initials: 60 Day_____/90Day:_____

60 Day Results/90 Day Results				
Treatment*	Dilutions of *M. bovis* BCG			
	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}
Primary Subculture (MPB)	/	/	/	/
Secondary Subculture ( ) + Ex. Neut.	/	/	/	/
Secondary Subculture ( ) + Ex. Neut.	/	/	/	/
Subculture Media (MPB) + Fresh Neut. Control	/	/	/	/
Subculture Media ( ) + Fresh Neut. Control	/	/	/	/
Subculture Media ( ) + Fresh Neut. Control	/	/	/	/
Subculture Media (MPB) Only Control	/	/	/	/
Subculture Media ( ) Only Control	/	/	/	/
Subculture Media ( ) Only Control	/	/	/	/
Uninoculated:				
Pr. Subculture Media (MPB) Negative Control	/			
Subculture Media ( ) Negative Control	/			
Subculture Media ( ) Negative Control	/			

*Fill in media type within the brackets "( )"
Pr. = Primary

SUMMARY OF RESULTS: Date/Initials:_____	
Bacteriostatic Effect Observed?	Yes_____ No_____
If yes, which treatment/media?	
Comments:	

Neutralization Confirmation Assay: Results Sheet for *M. bovis* (BCG)-Spray Products

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Product Name		Neutralizer	
Sample No.		Product Type (circle one)	Spray Liquid
Comments:			

TEST RESULTS: Date Recorded/Initials: 60 Day_____/90Day:_____

60 Day Results/90 Day Results

Treatment*	Dilutions of *M. bovis* BCG			
	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}
Primary Subculture (MPB)	/	/	/	/
Secondary Subculture ( ) + Ex. Neut.**	| / |	| / |	| / |	| / |
Secondary Subculture ( ) + Ex. Neut.**	| / |	| / |	| / |	| / |
Subculture Media (MPB) + Fresh Neut. Control	/	/	/	/
Subculture Media ( ) + Fresh Neut. Control**	| / |	| / |	| / |	| / |
Subculture Media ( ) + Fresh Neut. Control**	| / |	| / |	| / |	| / |
Subculture Media (MPB) Only Control	/	/	/	/
Subculture Media ( ) Only Control	/	/	/	/
Subculture Media ( ) Only Control	/	/	/	/
Uninoculated:				
Pr. Subculture Media (MPB) Negative Control	/			
Subculture Media ( ) Negative Control	/			
Subculture Media ( ) Negative Control	/			

*Fill in media type within the brackets "( )".
** There are two tubes per subculture medium; #1|#2/#1|#2 for 60 and 90 day results, respectively.

SUMMARY OF RESULTS: Date/Initials:_____

Bacteriostatic Effect Observed?	
If yes, which treatment/media?	
Comments:	

Neutralization Confirmation Assay: Serial Dilution/Plating Tracking Form for *M. bovis* (BCG)

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Neutralizer	
Sample No.		Organism Control #	
Sample No.		Comments:	

Confirmed by:_____	Dilution Tube							
	1	2	3	4	5	6	7	8
Vol. In Dil. Tube prior to Addition								
Volume Added to Dil. Tube								
Overall Dilution in Dil. Tube								
Volume Plated								
Overall Dilution on Plate								
Number of Plates per Dilution								
Media Plated Onto								
Comments:								

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Neutralization Confirmation Assay: Inoculum Enumeration Form for *M. bovis* (BCG)

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Organism	
Sample No.		Sample No.	

RESULTS: Date/Initials:_____			
Plating Method			
CFU per Dilution Plate			Average CFU per mL
Dilution	Plate 1	Plate 2	
1×10^{-5}			
1×10^{-6}			
1×10^{-7}			
1×10^{-8}			
TNTC = Too Numerous To Count			
Comments:			

Neutralization Confirmation Assay: Test Microbe Confirmation Sheet for *M. bovis* (BCG)

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Test Organism	
Sample No.		Comments:	

Source: Tube/Plate ID	Date/ Initials	Stain Results*	Media Information			Results	
			Type	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics

*Record Acid Fast or Gram Stain results as GPC=gram positive cocci, GNR=gram negative rods, AFR=acid fast rods, GPR=Gram positive rods.